



## Generation and characterization of human monoclonal neutralizing antibodies with distinct binding and sequence features against SARS coronavirus using XenoMouse<sup>®</sup>

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### Abstract

Passive therapy with neutralizing human monoclonal antibodies (mAbs) could be an effective therapy against severe acute respiratory syndrome coronavirus (SARS-CoV). Utilizing the human immunoglobulin transgenic mouse, XenoMouse<sup>®</sup>, we produced fully human SARS-CoV spike (S) protein specific antibodies. Antibodies were examined for reactivity against a recombinant S1 protein, to which 200 antibodies reacted. Twenty-seven antibodies neutralized 200TCID<sub>50</sub> SARS-CoV (Urbani). Additionally, 57 neutralizing antibodies were found that are likely specific to S2. Mapping of the binding region was achieved with several S1 recombinant proteins. Most S1 reactive neutralizing mAbs bound to the RBD, aa 318–510. However, two S1 specific mAbs reacted with a domain upstream of the RBD between aa 12 and 261. Immunoglobulin gene sequence analyses suggested at least 8 different binding specificities. Unique human mAbs could be used as a cocktail that would simultaneously target several neutralizing epitopes and prevent emergence of escape mutants.

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**Keywords:** Severe acute respiratory syndrome coronavirus; SARS-CoV; Human monoclonal antibodies

### Introduction

Coronaviruses (CoV) historically are known to cause relatively mild upper respiratory tract infections, and account for approximately 30% of the cases of the common cold in humans (Baker, 2004). However, a recently identified CoV, severe acute respiratory syndrome coronavirus (SARS-CoV), causes severe respiratory distress in humans leading to mortality in approximately 10% of infected individuals (Baker, 2004; Rota et al., 2003, [www.who.int/csr/sars/](http://www.who.int/csr/sars/)). In the year 2003, SARS-CoV established efficient human to human transmission resulting in several super-spreading events. By the end of the

outbreak in July of 2003, SARS-CoV was responsible for 774 deaths and 8096 cases worldwide involving 29 countries ([www.who.int/csr/sars/](http://www.who.int/csr/sars/)). Since the conclusion of the SARS outbreak, several reports in 2004 of confirmed cases of SARS of unknown origin indicate that the environmental threat of SARS-CoV still exists ([www.who.int](http://www.who.int), The Chinese SARS Molecular Epidemiology Consortium, 2004). SARS-CoV-like virus can be isolated from horseshoe bats in China, and researchers postulate that this is the natural reservoir for the virus (Lau et al., 2005; Li et al., 2005b). SARS-CoV-like virus remains present in intermediate wild animal hosts, such as the Himalayan palm civet, raising the possibility of re-emergence of SARS-CoV infection in humans (Chan et al., 2006; Peiris and Yuen, 2004). Because of the lingering threat, it is prudent to develop effective modalities of pre- and post-exposure treatments against SARS-CoV infection.

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During the SARS outbreak, although isolation measures proved effective in bringing the outbreak under control, a targeted and effective treatment for SARS-CoV remains highly desirable. In humans, SARS-CoV peak viral load is reached approximately about 10 days post onset of symptoms, thus offering an opportunity for effective post-exposure treatment (Chen et al., 2005, 2006). One modality of treatment that may limit virus replication and thus the spread of the virus is passive immunization with pre-formed neutralizing human monoclonal antibodies (mAbs). Such a treatment during the prodromal phase of the disease could aid in rapid clearance of virus and limit poor clinical outcome and person to person spread, without the adverse effects associated with use of corticosteroids, animal sera or human sera.

SARS-CoV mediates infection of target cells via the spike (S) protein expressed on the surface. SARS-CoV S protein is a type one transmembrane glycoprotein divided into two functional domains S1 (aa 15–680) and S2 (aa 681–1255) (He et al., 2004; Li et al., 2005a). The S1 domain mediates the interaction of the S protein with its cellular receptor, angiotensin-converting enzyme 2 (ACE2) (Li et al., 2003). A region of S1 consisting of 193 amino acids forms the receptor binding domain (RBD) which is responsible for ACE2 binding (Wong et al., 2004). More recently, a receptor binding motif (RBM) within the RBD, consisting of 70 amino acids, has been shown to directly contact the tip of ACE2 (Li et al., 2005a). The S2 domain of the S protein contributes to infection of the target cell by mediating fusion of viral and host membranes through a conformational change in which two conserved helical regions (HR1 and HR2) of the S protein are brought together to form a six-helix bundle fusion core (He et al., 2005a; Huang et al., 2002; Ingallinella et al., 2004; Tripet et al., 2004). Therapies that can disrupt interactions of these domains with the virus receptor will likely confer protection and be of therapeutic value.

The S protein serves as the main antigen that elicits protective immune responses, including neutralizing antibodies in infected humans and animals (Bisht et al., 2004; Buchholz et al., 2004; Chen et al., 2005; Greenough et al., 2005; He et al., 2005b; Hofmann et al., 2004). Intranasal or intramuscular application of a modified vaccinia virus Ankara (MVA) expressing S protein into mice elicits SARS-CoV neutralizing antibodies (Bisht et al., 2004). Immunization of mice with a DNA vaccine encoding the S sequence, devoid of the cytoplasmic domain and/or the transmembrane domain, results in the development of neutralizing antibodies as well as both CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses (Yang et al., 2004). However, it is the humoral (IgG) component of immunity and not the cellular component that inhibits viral replication (Yang et al., 2004). In fact, transfer of immune serum from immunized mice to naive mice reduces SARS-CoV titers following viral challenge (Subbarao et al., 2004). Together, these studies demonstrate that primarily Abs are responsible for protection against SARS-CoV replication, and indicate the potential therapeutic value of passive transfer of neutralizing Abs against SARS-CoV. The immunogenic property of the S protein, including its ability to induce neutralizing antibodies and its

essential role in viral attachment and fusion, make it an ideal target for developing effective immunotherapy against SARS-CoV infection.

Passive therapy with human immunoglobulin can confer immediate protection without the deleterious effects associated with the use of animal or chimeric Abs containing animal derived amino acid sequences (Lonberg, 2005). There are several ways to produce human mAbs; the method used here takes advantage of the transgenic XenoMouse® (Amgen British Columbia, Inc, Burnaby, BC). In the XenoMouse®, the mouse immunoglobulin (Ig) genes are replaced with genes encoding human Igs. Unlike using human B cells from infected or immunized individuals, these animals allow repeated immunization with the antigen of choice to induce B cells capable of producing a wide array of functionally relevant mAbs with high affinities against the antigen without further antibody engineering (Rathanaswami et al., 2005). This approach is highly flexible and allows extensive screening to select for different antibodies with unique properties. Furthermore, human mAbs can be directly produced from the hybridomas or the Ig genes can be cloned and used to generate recombinant cell lines, if necessary. Because of these advantages, we used the XenoMouse® to produce a large panel of neutralizing human mAbs against SARS-CoV S protein with distinct specificities.

## Results

### *Production of human neutralizing antibodies against S protein*

XenoMouse® IgG2κ animals were immunized with the ectodomain of S protein (Tor2) and hybridomas produced by standard protocol (Davis et al., 2004). Initially, hybridomas were grown to exhaustion and supernatants tested against a recombinant S protein S-V5-HIS (Tor2), and counter-screened against OVA-V5-HIS protein as a control. A large primary screen was followed by an additional secondary screen that resulted in the identification of 666 hybridomas capable of producing human antibodies (Abs) specific to SARS-CoV S protein with a minimum OD value of 0.3 (Table 1). These 666 Abs showed a wide range of binding efficiencies as determined by ELISA, many yielding saturating OD values of 4.0 and greater (Table 1). From the initial screening, the highest reacting 576 Abs were selected, further tested and characterized.

These 576 Abs were examined for their reactivity with the S1 domain of the S protein (Urbani) containing aa 12–672. This screening identified 200 Abs that were specific to the S1 domain of the S protein with reactivity ranging from 0.171 to 1.817 (Fig. 1). Samples with OD values greater than 2× the average background value (0.0825) were considered positive (Fig. 1).

### *Human Abs derived from XenoMouse® effectively neutralized SARS-CoV (Urbani)*

All S1 protein reactive Abs were tested for their ability to neutralize SARS-CoV in a microneutralization assay. Twenty-

Table 1  
Reactivity with the ectodomain of S protein (aa 12–1193)

OD values <sup>a</sup>	V5-S-HIS <sup>b</sup>	V5-OVA-HIS <sup>c</sup>
OD>0.0	1152	
OD>0.1	893	439
OD>0.2	739	94
OD>0.3	688	22
OD>0.4	657	11
OD>0.5	620	7
OD>0.6	593	6
OD>0.7	565	6
OD>0.8	541	6
OD>0.9	520	6
OD>1.0	508	4
OD>1.5	438	3
OD>2.0	401	3
OD>2.5	375	3
OD>3.0	352	2
OD>3.5	298	2
OD>4.0	136	0

<sup>a</sup> S positive supernatants with shown OD value or with greater.

<sup>b</sup> S positive supernatants accumulate from highest OD to lowest OD.

<sup>c</sup> Number of supernatants in each group that reacted with control V5-OVA-HIS.

seven of the strongly S1 reactive Abs showed complete neutralization (Table 2). As mentioned, the XenoMouse<sup>®</sup> was immunized with the full ectodomain of the S protein. Though this analysis has focused on the reactivity of Abs to S1, the remaining 376 S1 non-reactive Abs were examined in a microneutralization assay. An additional 57 Abs were found that completely neutralized 100TCID<sub>50</sub> SARS-CoV (Urbani) (data not shown). In addition, three Abs that reacted with the HR2 domain were examined in a microneutralization assay; however, they did not neutralize SARS-CoV (data not shown).

#### Differential reactivity of hybridoma supernatants with S1 fragments suggested several different regions of S protein recognized by human antibodies derived from XenoMouse<sup>®</sup>

Recombinant S1 proteins with a human IgG1 Fc tag (12–672, 12–510, 261–510, 318–510) were produced in 293T cells and purified to examine the differential binding and target region of the S1 reactive Abs (Fig. 2). These 200 S1 reacting Abs were further

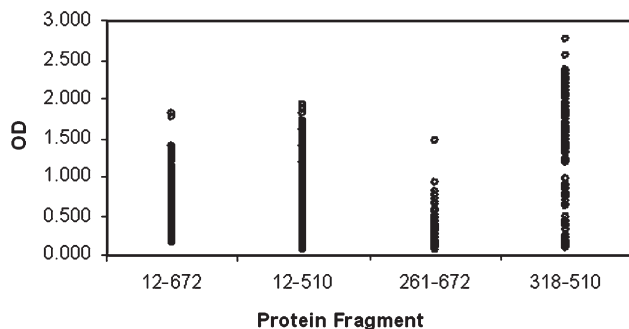


Fig. 1. Reactivity of HmAbs produced from hybridomas generated from immunized XenoMouse<sup>®</sup> against S1-Ig fragments by ELISA. All S-V5-HIS reactive Abs were tested against S1-Ig (12–672) coated recombinant protein. The 200 Abs that reacted with S1-Ig (12–672) were then tested for reactivity against three S1-Ig fragments (12–510, 261–510 and 318–510).

Table 2  
Likely binding region of human Abs

ELISA reactivity <sup>a</sup>	Group designation <sup>b</sup>	Number of Abs <sup>c</sup>	Number neutralizing <sup>d</sup>	Likely binding region <sup>e</sup>
All S1-IgG fragments	1A	13	9	318–510
12–672	3A	1	0	510–672
12–672, 12–510	2A	4	1	12–261
12–672, 12–510, 318–510	1B	45	14	318–510
12–672, 261–672	3B	2	0	510–672
12–510	2B	29	2	12–261
12–510, 318–510	1C	41	0	318–510
318–510	1D	29	1	318–510
Total		165	27	

<sup>a</sup> Fragments with which Abs react.

<sup>b</sup> Group designation determined based on likely binding region and fragment reactivity.

<sup>c</sup> Number of total Abs that reacted with designated S1-IgG fragments.

<sup>d</sup> Number of these Abs that completely neutralized 200TCID<sub>50</sub>-SARS-CoV (Urbani).

<sup>e</sup> Likely binding region of Ab group determined by differential fragment reactivity.

analyzed for their reactivity with additional S1 protein fragments by ELISA (i.e. 12–510, 261–672 and 318–510) (Fig. 1). The smallest fragment encoding the minimal RBD 318–510 often

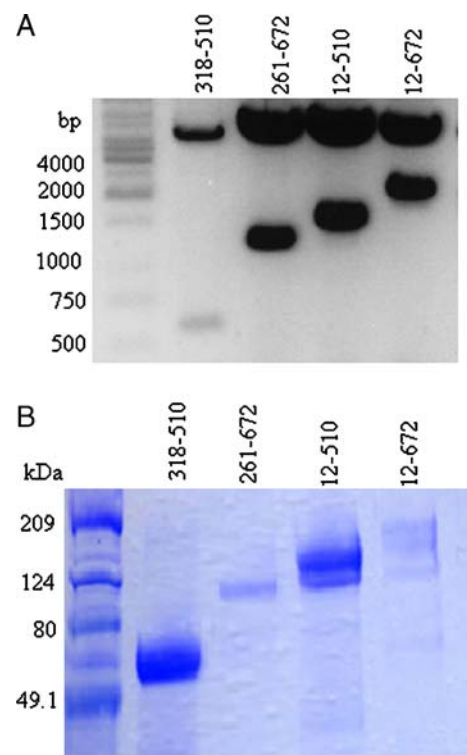


Fig. 2. Expression of overlapping fragments of the S1 domain of SARS-CoV S protein. (A) Four plasmid constructs encoding different fragments of the S1 protein (12–672, 12–510, 261–672, 318–510) were transformed into MC 1061/P3 cells and insert size confirmed by digestion with *NheI* and *BamHI* and analyzed on a 1% agarose gel. (B) Recombinant protein expression in transiently transfected 293T cells was confirmed by Coomassie Blue staining of a 4–20% SDS/PAGE gel.

yielded the highest reactivity for most of the Abs tested relative to the other fragments. Often 12–672 and 261–672 demonstrated the least reactivity, suggesting that the region between 511 and 672 could partially mask epitopes within 318–510 in these S1 fragments (Fig. 1).

Antibodies that yielded OD values above 0.5 (165 Abs that yielded approximately 6× above the background) were grouped based on their reactivity with various S1 fragments (Table 2). Comparison of the Ab reactivity across all S1 fragments indicated that most Abs reacted within the RBD 318–510. Abs that bound to 318–510 fell into four groups (group designation 1A–1D), based on their differential reactivity with the other S1 fragments (Table 2). The S1 fragments containing 511–672 possibly mask a dominant epitope(s) as represented by the high number of Abs found in groups 1C and 1D that do not yield high OD values with fragments containing this region. The difference in S1 fragment reactivity suggested that the epitope(s) recognized by Abs in groups 1C and 1D, which react with 12–510 and/or 318–510 fragments but lack reactivity with S1 fragments containing aa 511–672, are different than those of groups 1A and 1B which do react with fragments containing this region, though all group 1 Abs appear to bind within 318–510. Further epitope mapping was attempted using overlapping peptides derived from the 318–510 region of S1 domain (provided by NIH). These peptides consist of 18 amino acids with 10 amino acid overlap. None of the Abs showed significant reactivity with any of the peptides indicating that the antibodies recognized either conformational epitopes and/or required glycosylation.

Thirty-three Abs were found to likely react between 12 and 261 (i.e. groups 2A and 2B). These Abs did not react with 261–672 or 318–510; however, they do react with 12–510, and group 2A additionally reacts with 12–672 suggesting these Abs recognize an epitope(s) within the 12–261 region. In addition, a few Abs were found that react with the tail region of the S1 recombinant protein likely binding between 510 and 672. These Abs reacted solely with S1 fragments that included this tail region. The Abs in groups 2 and 3 have varying reactivity with the S1 fragments relevant to each group, which suggested that these Abs recognize a different epitope or have a varying affinity that is affected by the presence of 510–672 in group 2B as compared to 2A, and a lack of aa 12–261 in group 3A as compared to group 3B, which reacted to both 12–672 and 261–672 (Table 2). Mice were immunized with full-length S protein; therefore, Abs that did not react with the S1 domain were examined for their reactivity with HR1 and HR2 domains in the S2 region. None of the antibodies reacted with HR1 and three showed significant reactivity with HR2. Two of the three HR2 binding Abs resulted in high OD values (1.281 and 1.26) (data not shown).

Human Abs that recognized different regions of S1 varied in their neutralizing potential. A significant proportion of the S1 reactive neutralizing Abs reacted with the RBD, consistent with results obtained by other groups examining neutralizing Abs against SARS-CoV (Greenough et al., 2005; He et al., 2005a, b; He et al., 2004; Subbarao et al., 2004). A majority of neutralizing Abs fell into group 1A or 1B suggesting that these two groups recognize a dominant neutralizing epitope(s). On the contrary,

Abs that fall into groups 1C and 1D did not appear to recognize a significant neutralizing epitope(s) since only 1 neutralizing Ab out of 70 was identified between these groups (Table 2).

Three additional neutralizing Abs were found that most likely bind to a region between amino acids 12 and 261 of the S1 domain. This is analogous to other studies which have shown neutralizing ability of Abs that bind upstream of the known RBD (i.e. 130–150) (Greenough et al., 2005). Antibodies directed to important neutralizing epitope(s) accounted for approximately 16% of the total S1 specific Abs generated, therefore the majority of the Abs appear to recognize non-neutralizing epitope(s) within S1 (Table 2).

#### *Purification and characterization of neutralizing human monoclonal antibodies (mAbs) from XenoMouse® hybridomas*

Neutralizing mAbs were cloned by limiting dilution and confirmed by Ig gene sequencing. Twenty-four out of 27 previously identified neutralizing antibodies were recovered and purified by protein A/G affinity columns, 19 of these were subsequently confirmed as monoclonal by Ig gene sequencing. Following purification, the reactivity of mAbs was retested against the 318–510 fragment or 12–510, for those that failed to bind to 318–510. The high ELISA reactivity to the relevant S1-IgG fragments noted in the initial screening was maintained after the purification of the mAbs (Fig. 3). Most mAbs demonstrated a dose dependent binding in which OD values decreased with increasing dilution of the antibody. Other mAbs, such as 6B5 and 3H12, maintained high OD values possibly indicating a relatively higher affinity; however, the overall binding was somewhat lower than the other mAbs at the higher concentrations, possibly reflective of the availability of the epitope (Fig. 3).

Increasing dilutions of purified mAbs were tested for their ability to neutralize 200TCID<sub>50</sub> of SARS-CoV (Urbani). The neutralizing titer of the antibody was defined as the lowest concentration of mAb capable of completely neutralizing 200TCID<sub>50</sub> of SARS-CoV. The mAbs varied in their neutralizing potential. Some mAbs neutralized SARS-CoV at concentrations as low as 0.195 µg/ml. However, some mAbs were not able to neutralize virus below a concentration of 12.5 µg/ml (Table 3). This variance in neutralizing ability between mAbs may be due to differences in affinities, fine binding specificities and/or the extent of availability of the targeted epitope on virally expressed native S protein. Often, the degree of reactivity in ELISA did not correlate with the neutralization titer (Fig. 3 and Table 3). This suggested the possible limited availability of relevant epitopes in the virally expressed native S protein relative to their availability in a particular recombinant S1-Ig fragment, thus limiting neutralizing ability of certain mAbs.

#### *Immunoglobulin (Ig) gene sequences suggested different binding specificities among the human mAbs*

Each of the purified mAbs was sequenced, and previous group designations were further divided based on the immunoglobulin gene sequence data. Sequence analysis



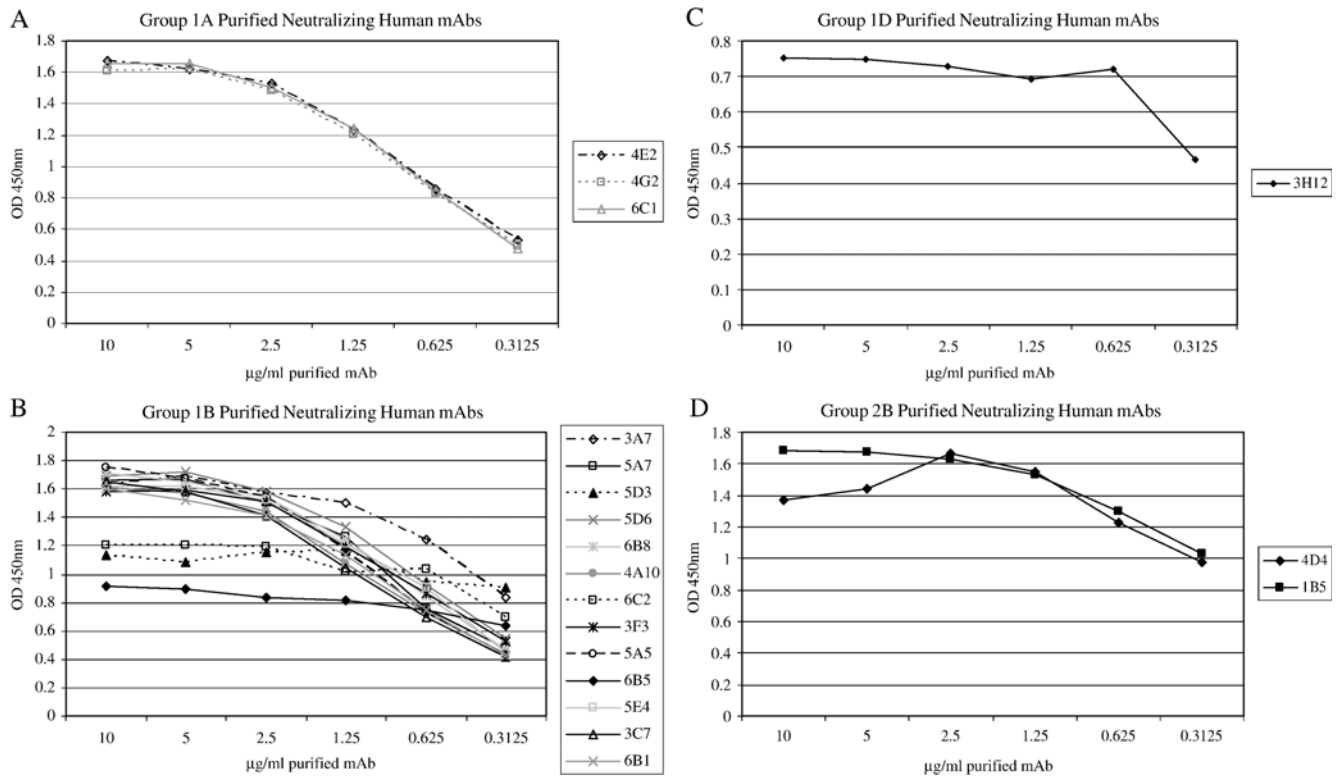


Fig. 3. Neutralizing mAbs were purified and examined for S1-Ig fragment reactivity in an ELISA. Recombinant proteins were coated on ELISA plates and increasing dilutions of mAb tested for reactivity to the relevant S1 recombinant protein. (A) Group 1A purified mAbs reactivity to 318–510 recombinant protein. (B) Group 1B purified mAbs reactivity to 318–510 recombinant protein. (C) Group 1D purified mAb reactivity to 318–510 recombinant protein. (D) Group 2B purified mAbs reactivity to 12–510 recombinant protein.

suggested that there were at least 8 different binding specificities among the panel of mAbs. Unique binding specificities were deduced from the usage of different V, J and D gene sequences, in the case of the heavy chain (H chain). There was preferential usage of A30 and JK4 rearrangement in the light chain (L chain); 14 of the 19 mAbs used this rearrangement for the L chain. The A30 V region was used 2 more times paired with different J segments. There was less repetition in the H chain rearrangements, though there was preferential usage of VH1–2, D3–10, JH4B rearrangement in the H chain, which is used 8 times, all with the A30 JK4 rearranged L chain. The next most frequent H chain rearrangement was VH1–18, D1–26, JH4B, all these also paired with the A30, JK4 rearrangement of the L chain. In addition, there were several more unique rearrangements on the H chain, usually paired with unique rearrangements of the L chain (Table 3). This analysis allowed the groups established earlier by ELISA reactivity to be further subdivided based on the assumption that the different gene rearrangements constitute the production of a unique binding site. Therefore, for example, Group 1B was divided into likely 4 different specificities based on different V(D)J usage in the H and L chains (Table 3). The first group, 1B1, has the V1–18, D1–26, JH4B H chain rearrangement paired with the A30, JK4 L chain and though the group 1B2 also uses the A30, JK4 rearrangement on the L chain, the H chain rearrangement was different, VH1–2, D3–10, JH4B; this likely results in the recognition of a different epitope between groups 1B1 and 1B2

(Table 3). The remaining members of the 1B group, 1B3 and 1B4, had unique H and L chain rearrangements and so likely recognize their own unique epitopes (Table 3). The differences in H chain and L chain rearrangements are important because they influence the structure of the CDR3 region which confers binding specificity.

Different rearrangements resulted in different CDR3 region sequences in both the H and L chains (Fig. 4 and Table 3). Our data demonstrated sequence differences within the CDR3 regions of several mAbs. The CDR3 region of group 1A1 and 1B2 is the same except for an S in the fourth position of the H chain in group 1A1 rather than the germline encoded T as in group 1B2 (Fig. 4 and Table 3). This change can alter the binding specificity or affinity between these two groups of mAbs, therefore the specific identification of the recognized epitope is required to delineate any differences between these groups of mAbs.

Two groups of mAbs showed changes in the CDR1 and CDR2 regions. For example, 4G2 and 6C1 have three amino acid differences within the CDR1 as compared to 4E2 although they all fall into group 1A1 and have the same V(D)J usage (Fig. 4). The mAb 6B1 has a single amino acid change in the CDR2 region when compared to 3C7; again this change may alter the affinity of 6B1 for the binding region within the S protein resulting in slightly better neutralizing ability noted with 6B1 (Fig. 4). In addition, several mAbs have changes in one or more of the four framework regions making up the structure of

Table 3  
Summary of HmAbs reactivity, neutralizing titer and heavy (H) and light (L) chain usage

HmAb	Group <sup>a</sup>	Reactivity S1-IgG (12–672, 12–510, 261–672, 318–510) <sup>b</sup>	Binding region <sup>c</sup>	Neutralizing titer 200TCID <sub>50</sub> (μg/ml) <sup>d</sup>	H chain <sup>e</sup>	L chain <sup>e</sup>	H CDR3 <sup>f</sup>	L CDR3 <sup>f</sup>
4-E2	1A1	0.919, 1.518, 0.551, 1.839	318–510	0.781	VH1-2, D3-10, JH4B	A30, JK4	GPHSFGSGSYFPDY	QQYNSYPLT
4-G2	1A1	1.200, 1.662, 0.665, 1.811	318–510	0.781	VH1-2, D3-10, JH4B	A30, JK4	GPHSFGSGSYFPDY	QQYNSYPLT
6-C1	1A1	1.226, 1.586, 0.649, 2.405	318–510	0.781	VH1-2, D3-10, JH4B	A30, JK4	GPHSFGSGSYFPDY	QQYNSYPLT
3-A7	1B1	1.307, 1.523, 0.379, 1.964	318–510	0.195	VH1-18, D1-26, JH4B	A30, JK4	GRYLDY	LQYNSYPLT
5-A7	1B1	1.111, 1.449, 0.366, 1.997	318–510	0.781	VH1-18, D1-26, JH4B	A30, JK4	GRYLDY	LQYNSYPLT
5-D3	1B1	0.968, 1.316, 0.403, 2.020	318–510	0.195	VH1-18, D1-26, JH4B	A30, JK4	GRYLDY	LQYNSYPLT
5-D6	1B1	0.747, 1.313, 0.355, 2.117	318–510	0.195	VH1-18, D1-26, JH4B	A30, JK4	GRYLDY	LQYNSYPLT
6-B8	1B1	1.045, 1.704, 0.497, 2.133	318–510	0.781	VH1-18, D1-26, JH4B	A30, JK4	GRYLDY	LQYNSYPLT
4-A10	1B2	1.013, 1.524, 0.567, 1.792	318–510	3.125	VH1-2, D3-10, JH4B	A30, JK4	GPHTFGSGSYFPDY	QQYNSYPLT
6-C2	1B2	1.005, 1.603, 0.586, 1.849	318–510	0.781	VH1-2, D3-10, JH4B	A30, JK4	GPHTFGSGSYFPDY	QQYNSYPLT
3-F3	1B2	1.075, 1.349, 0.325, 1.887	318–510	0.781	VH1-2, D3-10, JH4B	A30, JK4	GPHTFGSGSYFPDY	QQYNSYPLT
5-A5	1B2	0.986, 1.187, 0.337, 2.310	318–510	0.195	VH1-2, D3-10, JH4B	A30, JK4	GPHTFGSGSYFPDY	QQYNSYPLT
6-B5	1B2	1.040, 1.324, 0.430, 2.087	318–510	3.125	VH1-2, D3-10, JH4B	A30, JK4	GPHTFGSGSYFPDY	QQYNSYPLT
5-E4	1B3	0.735, 1.199, 0.298, 2.275	318–510	12.5	VH1-2, N/A, JH4B	A30, JK5	GRYLDY	LQYNSYPIT
3-C7	1B4	1.092, 1.422, 0.357, 2.193	318–510	12.5	VH3-33, D2-2, JH4B	L5, JK4	DPLGYCSSTSCSYFDY	QQANNFPLT
6-B1	1B4	1.128, 1.166, 0.369, 2.093	318–510	3.125	VH3-33, D2-2, JH4B	L5, JK4	DPLGYCSSTSCSYFDY	QQANNFPLT
3-H12	1D	0.185, 0.318, 0.090, 1.304	318–510	3.125	VH4-59, D3-9, JH6B	A30, JK3	DYDILTGYSNYYGMDV	LQHNSYPFT
4-D4	2B1	0.258, 0.761, 0.103, 0.101	12–261	12.5	VH3-33, D4-17, JH4B	A1, JK2	GGDGERFDY	MQGTHWPPYVQ
1-B5	2B2	0.463, 1.292, 0.110, 0.191	12–261	0.195	VH3-33, N/A, JH5B	A30, JK4	GDFYWFDP	QQYNSYPLT

<sup>a</sup> Group designation based on fragment reactivity demonstrated previously and CDR3 sequence data.

<sup>b</sup> OD values obtained for each mAb against indicated fragments during the original screen.

<sup>c</sup> Likely binding region as determined previously by S1-IgG fragment reactivity.

<sup>d</sup> Concentration of mAb that completely neutralizes 200TCID<sub>50</sub> SARS-CoV (Urbani).

<sup>e</sup> Ig gene segment usage.

<sup>f</sup> CDR3 sequences of each mAb.

the mAb (data not shown). Though the CDR regions form the antigen binding pocket, changes within the framework region can also contribute to binding specificity and affinity. Therefore, these changes could result in a greater separation of the mAb groups based on CDR regions and result in different specificity for S protein or binding affinity for the same region. For example, 5D6 belongs to the 1B1 group; however, 5D6 carries a mutation in the framework 3 region, which could potentially alter the recognition of S protein by this mAb and therefore set it apart from the other members of the same group. Monoclonal Abs 6B1 and 3C7 both belong to group 1B4; however, 3C7 has a single amino acid change in framework region 3 which may influence binding specificity and affinity, and therefore, influence neutralizing capability (data not shown).

## Discussion

Consistent with findings of other groups, we have shown that immunization of XenoMouse<sup>®</sup> with recombinant S protein resulted in the production of SARS-CoV specific neutralizing Abs (Berry et al., 2004; Bisht et al., 2004; Buchholz et al., 2004; Greenough et al., 2005; He et al., 2005a; He et al., 2004; Hofmann et al., 2004). Moreover, we were able to use B cells from these mice to generate a panel of neutralizing human mAbs against SARS-CoV. The majority of our S1 reactive mAbs reacted with the RBD (318–510) and likely neutralized virus by blocking SARS-CoV S protein binding to ACE2 (Li et al., 2003; Sui et al., 2004). Therefore, it is apparent that mAbs produced in the current study recognize a critical domain not

only involved in the induction of an effective immune response against SARS-CoV infection but also in viral attachment (He et al., 2004; He et al., 2005a, b).

The Ig sequence analysis indicated that there was a preferential usage of certain H and L chain genes namely, V1–2 D3–10, JH4B and A30 JK4. There were several mAbs that reacted with the S1 fragment but exhibited considerable diversity in their H and L chain gene segment usage. This suggested that these mAbs possibly recognized distinct epitopes within a given domain. A majority of the neutralizing mAbs that bound to 318–510 fell into groups 1A and 1B and exhibited a similar S1 fragment binding pattern. However, the Ig sequence data suggest that there could be up to 5 different binding specificities within these two groups. For example, group 1B, whose likely binding region is 318–510, could be divided into 4 different binding specificities based on different V(D)J rearrangements that yield unique CDR3 regions. In addition, variations seen within the CDR3 sequences of mAbs with common Ig gene usage, such as those seen between groups 1A1 and 1B2, suggested that these mAbs may bind to different epitopes or bind the RBD epitope with varying affinity. Based upon the Ig sequence analysis, we speculate that at least 8 different binding specificities may be represented in our panel of mAbs; when considering mutations found in CDR1, CDR2 and framework regions, this could increase the number of different S1 specificities. The contribution of framework regions to Ab binding is illustrated in the generation of a chimeric antibody to respiratory syncytial virus (RSV) or TNF-α. When the CDR regions of neutralizing murine mAbs were transferred to the

A				B			
H Chain	CDR1	CDR2	CDR3	L chain	CDR1	CDR2	CDR3
<b>VH1-18, D1-26, JH4B</b>				<b>A1, JK2</b>			
Germline	GYTFTSYGIS	WISAYNGNTNYAQKLQG	GRYLDY	Germline	RSSQSLVYSDGNTYLN	KVSNWDS	
5D6	-----	-----	-----	4D4	-----R-----F--	-----	MQGTHWPPYVQ
5D3	-----	-----	-----	<b>A30, JK1</b>			
6B8	-----	-----	-----	Germline	RASQGIRNDLG	AASSLQS	LQYNSYPWT
3A7	-----	-----	-----	2B10	-----	-----	-----
5A7	-----	-----	-----	<b>A30, JK4</b>			
<b>VH1-2, N/A, JH4B</b>				Germline	RASQGIRNDLG	AASSLQS	LQHNSYPLT
Germline	GYTFTGYMH	WINPNSGGTNYAQKFQG	GTLYDY	5D6	---D---	-----	--Y-----
5E4	-----V-	-----	-R----	5D3	---D---	-----	--Y-----
<b>VH1-2, D3-10, JH4B</b>				6B8	---D---	-----	--Y-----
Germline	GYTFTGYMH	WINPNSGGTNYAQKFQG	GPHTFGSGSYPFDY	3A7	---D---	-----	--Y-----
3F3	-----	-----	-----	5A7	---D---	-----	--Y-----
6C2	-----	-----	-----	4A10	-----S-	-----	Q-Y-----
6C1	-FP--D---	---S-----	---S-----	5A5	-----S-	-----	Q-Y-----
4G2	-FP--D---	---S-----	---S-----	3F3	-----S-	-----	Q-Y-----
4E2	-----	---S-----	---S-----	6C2	-----S-	-----	Q-Y-----
5A5	-----	-----	-----	1B5	-----S-	-----	Q-Y-----
4A10	-----	-----	-----	6C1	-----S-	-----	Q-Y-----
6B5	-----	-----	-----	4G2	-----S-	-----	Q-Y-----
<b>VH3-33, N/A, JH5B</b>				6B5	-----S-	-----	Q-Y-----
Germline	GFTFSSYGMH	VIWYDGSNKYYADSVKG	GDFYWFDP	4E2	-----S-	-----	Q-Y-----
1B5	-----	-----	-----	<b>A30, JK5</b>			
2B10	-----	-----	-----	Germline	RASQGIRNDLG	AASSLQS	LQHNSYPIT
<b>VH3-33, D2-2, JH4B</b>				5E4	-----	-----	--Y-----
Germline	GFTFSSYGMH	VIWYDGSNKYYADSVKG	DPLGYCSSTSCSYFDY	1G3	-----	-----	--Y-----
6B1	-----[-	-----FH-----	-----	<b>L5, JK4</b>			
3C7	-----[-	-----F-----	-----	Germline	RASQGISSWLA	AASSLQS	QQANSF##T
<b>VH3-33, D4-17, JH4B</b>				6B1	-----N--	-----	---N-PL-
Germline	GFTFSSYGMH	VIWYDGSNKYYADSVKG	GGDGERFDY	3C7	-----N--	-----	---N-PL-
4D4	-----	-----	-----				
<b>VH4-59, D3-9, JH6B</b>							
Germline	GGSISSYYWS	YIYYSGSTNYNPSLKS	DYDILTGYSNYYGMDV				
3H12	D-----F--	-----	-----				

Fig. 4. Alignment of CDR sequences of neutralizing mAbs. Immunoglobulin genes of neutralizing antibodies were sequenced. Alignment of the amino acid sequences of the heavy chain variable region (A) and light chain variable region (B) of all mAbs are depicted and arranged by common gene segment usage. Additions in mAb sequences not contained in germ line sequence are annotated (#) in germ line sequence. N/A specific gene segment could not be identified.

framework regions of human IgG1, the resulting chimeric mAbs lost their representative neutralizing abilities (Tempest et al., 1991, 1994). Therefore, the potential for changes that we saw in the CDR and framework regions of our SARS S1 specific mAbs may result in differences in binding specificity or affinity; however, detailed epitope mapping is required to fully characterize the fine binding specificity each of the mAbs.

In addition to antibodies that bind to the RBD, we isolated other neutralizing mAbs (groups 2A and 2B) that can interact with epitopes contained within amino acids 12–261, upstream of the RBD. The presence of these neutralizing epitopes demonstrates an important, yet unclearly defined role of this region in SARS-CoV infection. Since this region has not been implicated in binding of ACE2, these mAbs may prevent S protein from acquiring the conformation required for mediating fusion with the target membrane. Deletions within the first 300 aa of S1 eliminate fusion ability of S protein in a cell-to-cell fusion assay while maintaining receptor binding function (Xiao et al., 2004). The N-terminal region of S protein also seems to play a role in the trimerization of S protein, a structure that may be required for fusion (Xiao et al., 2004). Therefore, binding of mAb to this region may interfere with important steps that facilitate fusion. Further functional characterization of these mAbs could yield information on the function of this region of SARS-CoV S protein in viral entry.

Screening of full-length S reactive, but S1 non-reactive, Abs resulted in the identification of 57 additional neutralizing Abs. These Abs likely react at the junction between S1 and S2, within the S2 domain, or recognize important conformational epitopes that require the entire S protein. Since the S2 domain is involved in fusion of the viral and cellular membranes, antibodies specific for regions within the S2 domain likely neutralize by inhibiting fusion. Such antibodies will be of value to more carefully study the fusion events. Moreover, the S2 domain is more highly conserved than the S1 domain, and therefore S2 region reactive mAbs can be combined with mAbs with distinct S1 binding specificities that can prevent viral attachment to create a mixture of antibodies with a broader range of specificity to more effectively prevent infection by a wide range of clinical isolates (Rota et al., 2003; Zhang et al., 2004). Additionally, such an approach would significantly reduce or eliminate the possibility of generating antigenic variants that might more readily arise when a single mAb is used.

The mAbs produced in this study are unique in that they express both human heavy and light chains. If proven to be of therapeutic value, these antibodies will have a number of added advantages. The IgG<sub>2</sub> antibodies will be readily available in extracellular body fluids (a known property of the IgG<sub>2</sub> antibodies), IgG<sub>2</sub> antibodies fail to activate the classical



complement pathway, and since there is essentially no Fc receptor (FcR) for IgG<sub>2</sub> on macrophages and other phagocytes to which antibodies can bind and mediate their functional effects, they are unlikely to facilitate antibody dependent enhancement (ADE) of viral infection through FcRs.

The immunogenicity of non-human derived Abs or chimeric Abs with animal sequences could result in their rapid clearance and reduce their efficacy (Lin et al., 2005). In addition, adverse reactions, like those seen with chimeric or Abs from different species, could be avoided by using human mAbs. Further, the human mAbs are structurally intact and thus retain full complement of their functional properties.

During an outbreak, the SARS-CoV can mutate and exhibit antigenic variation. In fact sequence analysis indicates that the clinical isolates could be divided into early, middle and late isolates (Sui et al., 2005). The significance of this is demonstrated in the ability of later isolates to escape neutralization by a mAb that effectively neutralized an earlier isolate (Yang et al., 2005). Therefore, it is important to produce neutralizing mAbs that are effective against a wide range of clinical isolates with antigenic diversity. Because of the potential evolution of antigenic variants, an effective passive therapy against SARS-CoV will likely contain a cocktail of neutralizing mAbs that target different epitopes and/or steps in the entry process, such as blocking receptor binding and fusion. We believe that several of our mAbs can be used to prepare such a cocktail for potential therapeutic use. In addition, identifying neutralizing epitopes conserved over a range of clinical isolates has significant implications for developing a more effective vaccine largely devoid of non-neutralizing epitopes (Traggiai et al., 2004).

Antibodies have proven important in the immune response against SARS-CoV (Bisht et al., 2004; Buchholz et al., 2004; Chen et al., 2005; Greenough et al., 2005; He et al., 2005b; Hofmann et al., 2004; Zhang et al., 2006). Recovered patients showed a higher Ab titer and neutralizing Abs against S protein were maintained (Zhang et al., 2006). Therefore, utilizing human mAbs is a viable therapeutic option for the treatment of SARS-CoV infection. Abs that are capable of neutralizing *in vitro* usually can also confer *in vivo* protection against a viral challenge by reducing viral titer (Chen et al., 2005; Greenough et al., 2005; Sui et al., 2004, 2005; Yang et al., 2005). Therefore, these mAbs may be used for passive immunotherapy to provide instantaneous protection to individuals infected with the SARS-CoV in the case of re-emergence of SARS-CoV in the human population.

## Materials and methods

### Virus and cells

SARS coronavirus (SARS-CoV) Urbani strain was obtained from CDC. Virus was propagated in Vero cells in OptiPro serum free medium (SFM) (Gibco, Carlsband, CA). The TCID<sub>50</sub> value was then determined by infecting  $5 \times 10^3$  Vero cells/well in a 96 well plate with serial 1:10 dilutions of SARS-CoV. After 3 days of incubation at 37 °C in a 5%CO<sub>2</sub> humidified incubator, cells

were evaluated for cytopathic effect (CPE). The TCID<sub>50</sub> value was calculated by the Reed-Muench method.

### Expression of S proteins

A cDNA encoding amino acids 1–1193 of the ectodomain of the S protein (Tor2) (kind gift from Marco Marra and Caroline Astell at the British Columbia Cancer Agency Genome Sciences Centre) was cloned into the BaculoDirect™ Baculovirus Expression System (Invitrogen, Carlsband, CA) in frame with a V5-HIS-tag. The protein was expressed in Sf9 cells and purified using Probond™ Nickel-Chelating Resin (Invitrogen, Carlsband, CA).

The S1-IgG (Urbani) fragments used in screening and domain mapping consisted of amino acids 12–672, 12–510, 261–672 or 318–510, in addition to the C5 signal sequence and a human IgG Fc lacking the transmembrane domain (Wong et al., 2004). The expression plasmids encoding different S1-Ig fragments (i.e. aa 12–672, 12–510, 261–672, 318–510) were transformed into MC1061/P3 ultracompetent cells (Invitrogen, Carlsband, CA) and selected on tetracycline and ampicillin agar plates. The constructs were confirmed by restriction analysis using *NheI* and *BamHI*. The cDNAs encoding S1-Ig fragments were transfected into 293T cells using a CaPO<sub>3</sub> transfection kit (Invitrogen, Carlsband, CA). Briefly, 293T cells were seeded 1 day prior to transfection, and the medium was changed the following morning. The CaPO<sub>3</sub> transfection procedure was performed as follows: 10 µg DNA + 50 µl CaCl<sub>2</sub> (2 M) + 450 µl sterile H<sub>2</sub>O was mixed and added dropwise to 500 µl of HBS while aerating (values are per transfected plate). Next day, the cells were washed with PBS + 1 mM CaCl<sub>2</sub> + 0.5 M MgCl<sub>2</sub> and medium replaced with 293T SFM supplemented with 2 mM L-glutamine and antibiotic/antimycotic (Gibco, Carlsband, CA). Cells were incubated at 37 °C for an additional 24 h at which time the medium was harvested and protease inhibitor tablets added (Roche Diagnostics, Indianapolis, IN). The supernatant was spun at 1500 rpm for 5 min, and the supernatant was mixed with Protein-A sepharose beads (Santa Cruz Biotechnology, Santa Cruz, CA) by rocking overnight at 4 °C. The beads were then washed with PBS + CaCl<sub>2</sub> + MgCl<sub>2</sub> + 0.5 M NaCl one time followed by two additional washes with PBS + CaCl<sub>2</sub> + MgCl<sub>2</sub>. Protein was eluted using 50 mM sodium citrate/50 mM glycine at pH 2 and neutralized immediately using Tris–HCl (pH 9.5). Protein was concentrated on Centricon filters (Millipore, Bedford, MA) spinning at 3000 rpm for 1 h at 4 °C and dialyzed overnight at 4 °C against PBS. Protein production was verified by SDS-PAGE followed by Coomassie Blue (BioRad, Hercules, CA) staining (Wong et al., 2004). The identity of the protein was confirmed by western blotting using pooled sera obtained from mice immunized with inactivated virus.

### Generation of hybridomas from S protein immunized Xenomouse®

Five micrograms of purified S protein was emulsified in Titermax Gold adjuvant (Sigma, Oakville ON) and 6–10 week old IgG<sub>2</sub>κ XenoMouse® animals were immunized



intraperitoneally. Subsequent boosts were performed sequentially using TiterMax Gold or alum (Sigma, Oakville ON) as adjuvants. When the animals developed an anti-S antibody response, a final boost in PBS was performed, 4 days later the spleen and lymph cells were harvested, fused with P3 myeloma cells and HPRT+hybridomas were selected in hypoxanthine-azaserine (HA) using a standard protocol (Davis et al., 2004).

#### Antibody screening

Hybridoma supernatants from a total of 11,520 wells were individually screened for S reactivity by ELISA against S-V5-HIS. A secondary screen of 1152 wells against S-V5-HIS with a counter screen against OVA-V5-HIS as a negative control was performed. Hybridoma supernatants yielding OD values above ~0.7 when tested against S-V5-HIS (Tor2) were further tested against various S1-Ig fragments by ELISA. Initial screening was carried out using the full-length S1-Ig (12–672). Briefly, plates were coated with 50 ng/well of S1-IgG protein overnight at 4 °C. The plates were blocked using 5% non-fat milk, 0.05% Tween-20 for 1 h at room temperature, washed and 50 µl of hybridoma supernatant (diluted 1:3.5) was added to each well and incubated at room temperature for 1 h. After washing, 50 µl/well of HRP conjugated goat-anti-human antibody was added and incubated for 1 h at room temperature. Following washing, the antibody binding was detected using 50 µl/well of substrate (BD OptEIA, BD Biosciences Pharmingen, San Diego, CA) and the reaction stopped using 25 µl of 10% HCl. The absorbance was then read using a microplate reader (BioRad, Hercules, CA) at 450 nm. The same procedure was followed for screening of hybridoma supernatants (used at a dilution of 1:6) and purified human monoclonal antibodies (mAbs) using the other S1-Ig fragments.

#### Neutralization assay

The neutralizing ability of hybridoma supernatants against SARS-CoV (Urbani) was tested using a microneutralization assay. Vero cells were seeded at  $5 \times 10^3$  cells per well in a 96 well plate in OptiPro SFM (Gibco, Carlsband, CA) a few hours prior to the neutralization assay. Neutralizing ability of the Abs in hybridoma supernatants was tested by mixing 50 µl of hybridoma supernatant, diluted 1:2 in 50 µl of OptiPro SFM (Gibco, Carlsband, CA) with 200TCID<sub>50</sub> of virus in 100 µl of medium for 1 h at 37 °C. Following incubation, 100 µl of the antibody/virus mixture was added in duplicate to Vero cells and incubated at 37 °C for 3 days. At this time, cells were visually observed for cytopathic effect (CPE; indicated by rounding of Vero cells) as an indicator of SARS-CoV infection. The same assay was performed using 1:4 serial dilutions of purified human monoclonal antibodies (mAbs).

#### Purification of human mAbs

Hybridomas that were positive for the production of neutralizing antibodies were cloned by limiting dilution and

the clones cultured to produce large quantities of mAbs. Supernatants from these hybridomas were purified using Protein A/G affinity columns.

#### Sequencing of human mAbs

Total RNA was purified from approximately  $10^5$  hybridoma cells using an RNeasy Mini Kit (Qiagen, Mississauga, ON) as per the manufacturer's instructions. The PCR amplification protocol and primers have previously been described (Gallo et al., 2000; Marks et al., 1991). V family primers were pooled or used individually. Sequencing was performed by Lone Star Labs (Houston, TX) using the BigDye™ Terminator Version 3.0 DNA sequencing kit (Applied Biosystems, Foster City, CA) and ABI 3730 or 3100 automated sequencers (Applied Biosystems, Foster City, CA).

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